

Abnormal Retinal Development in the *Btrc* Null Mouse

Mark Baguma-Nibasheka and Boris Kablar*

Department of Anatomy and Neurobiology, Dalhousie University Faculty of Medicine, Halifax, Canada

Abstract

Previous microarray analysis revealed *beta-transducin repeat containing (Btrc)* down-regulation in the retina of mouse embryos specifically lacking cholinergic amacrine cells (CACs) as a result of the absence of skeletal musculature and fetal ocular movements. To investigate the role of *Btrc* in the determination of retinal cell fate, the present study examined retinal morphology in *Btrc*^{-/-} mouse fetuses. The *Btrc*^{-/-} retina showed a normal number of cell layers and number of cells per layer with normal cell proliferation and apoptosis. However, there was a complete absence of CACs and a decrease in tyrosine hydroxylase-expressing amacrine cells. The population of other amacrine cell subtypes was normal, whereas that of the precursor cells was decreased. There was also a reduction in the number of retinal ganglion cells, whereas their progenitors were increased. These findings suggest a role for *Btrc* in regulating the eventual ratio of resulting differentiated retinal cell types.

Keywords

Btrc mutant mouse; retinal differentiation; amacrine cells; beta-TrCP

INTRODUCTION

The differentiation of the mammalian retina is subject to various intrinsic and extrinsic factors that determine the fate of individual progenitor cells and the precise ratios of the resulting cell types. Many studies have particularly implicated the progenitor cells' genetic identity in this process (Dyer and Cepko, 2000; Marquardt et al., 2001; Dyer et al., 2003; Li et al., 2004; de Melo et al., 2005; Fujitani et al., 2006), whereas others have demonstrated a distinct influence by the environment as well. Specifically, Belliveau and Cepko (1999) and Cepko (1999) revealed that the cellular microenvironment affects the specification of retinal progenitor cell fate, and previous work in our laboratory showed that mechanical forces also affect retinal morphogenesis and cell diversity (Kablar, 2003). The retina of *Myf5*^{-/-}:*MyoD*^{-/-} mouse embryos, which, due to the lack of striated muscle, exhibit no fetal ocular movements (FOMs), was found to have disturbances in the number of amacrine and ganglion cell types and subtypes and their precursors. In particular, in the retina of these amyogenic embryos, there was a complete absence of cholinergic amacrine cells (CACs) (Kablar, 2003), known to be important in potentiating the response of retinal ganglion cells

*Correspondence to: Boris Kablar, Department of Anatomy and Neurobiology, Dalhousie University Faculty of Medicine, 5850 College Street, Halifax, NS, Canada B3H 1X5. bkablar@dal.ca.

(RGCs) to moving stimuli (He and Masland, 1997, Yoshida et al., 2001). Since the signal transduction pathways translating mechanical stimuli to meaningful molecular instructions for terminal retinal cell differentiation are still unclear, we subsequently used microarray analysis to help identify genes that might be important in the regulation of CAC differentiation for the functional maturation of the visual system and its eventual ability to perform motion vision. That analysis revealed a down-regulation of several genes, including *beta-transducin repeat containing (Btrc)*, in the retina of the amyogenic embryos (Baguma-Nibasheka et al., 2006).

Btrc encodes the beta-transducin repeat containing protein (β -TrCP), a member of the E3 ubiquitin ligase family of proteins that contain F-box and WD40 repeats (Maniatis, 1999; Kipreos and Pagano, 2000; Jin et al., 2004). As subunits of the Skip1/Cullin/F-box (SCF) enzyme complex, F-box proteins are required for the conjugation of ubiquitin to the lysine residues of particular intracellular proteins, thereby targeting those proteins as substrates for lysis by the 26S proteasome (Koepp et al., 1999; Kipreos and Pagano, 2000; Cardozo and Pagano, 2004; Ang and Wade Harper, 2005; Petroski and Deshaies, 2005). Numerous studies have shown that, by promoting the catabolism or processing of a broad range of proteins and transcription factors at specific stages of the cell cycle, the SCF $^{\beta$ -TrCP complex is involved in the control of such diverse processes as: (1) chromosome stabilization (REST; Guardavaccaro et al., 2008), (2) mitotic progression (Cdc25a, Emi1, and Bora; Busino et al., 2003; Margottin-Goguet et al., 2003; Seki et al., 2008), (3) cell proliferation and apoptosis (Shh and IkappaB; Jensen and Wallace, 1997; Hatakeyama et al., 1999; Hattori et al., 1999; Winston et al., 1999), (4) cell-autonomous circadian oscillation (PERIOD2; Guardavaccaro and Pagano, 2006; Reischl et al., 2007), and (5) embryonic patterning (Gli3; Wang and Li, 2006), as well as, (6) cell adhesion and decidualization (β -catenin; Kitagawa et al., 1999; Herington et al., 2007; Seki et al., 2008).

Btrc is known to be overexpressed and oncogenic in epithelial tumors (Saitoh and Katoh, 2001, Kudo et al., 2004, Noubissi et al., 2006), a fact that may relate to its crucial involvement in the control of intracellular levels of the tumor suppressor REST (Westbrook et al., 2008). The absence of β -TrCP, on the other hand, leads to chromosome misalignment, hyperploidy, and increased apoptosis (Guardavaccaro et al., 2003, Nakayama et al., 2003) and, in vivo, to impaired spermatogenesis and defective mammary gland development (Guardavaccaro et al., 2003, Kudo et al., 2004).

Interestingly, in a cDNA microarray analysis of gene expression in the retina of mouse embryos specifically lacking CACs as a result of the absence of skeletal musculature and consequently of FOMs, we found that *Btrc* mRNA was 5-fold down-regulated in the amyogenic embryos (Baguma-Nibasheka et al., 2006). Furthermore, perhaps because it facilitates the degradation of REST and Shh (which, according to Chong et al., 1995 and Ingham and McMahon, 2001, may inhibit neuronal gene expression programs), β -TrCP has been shown to be essential for proper final differentiation of neurons (Westbrook et al., 2008). It is, therefore, conceivable that the *Btrc* gene and/or its protein product β -TrCP might play some role in regulating the pattern of cell differentiation and the consequent functional maturation of the retinal neuronal cell types. In this study, therefore, we examined the retina of *Btrc*^{-/-} mouse at the end of gestation, where our previous research on retinal

dysplasia due to the absence of FOMs has revealed differences from normal retinal development.

RESULTS

Spatial Organization of the Retinal Layers Is Not Affected by the *Btrc* Null Mutation

To investigate the effect of *Btrc* deletion on the formation of retinal layers and the generation of the correct cell numbers within those layers, we examined the basic laminar organization in hematoxylineosin stained meridional sections of the retina. An inner limiting membrane was identifiable on the inner surface of the retina in both the control wild-type embryos and their *Btrc*^{-/-} littermates (Fig. 1), and the neural retina was clearly delineated into the inner and outer nuclear (or neuroblastic) layers (INL and ONL, respectively; Fig. 1). At this stage, neural retina still consists of only two nuclear layers, but, in a day or two, it will clearly consist of three nuclear layers (ganglion cell layer or GCL, INL, and ONL). ONL is destined to form primarily the photo-receptors, while INL is destined to form principally the ganglion cells (Kaufman, 1999). The outer surface of the retina was lined by the retinal pigment epithelium (RPE), with normal melanin pigmentation in the RPE of both the controls and the *Btrc*^{-/-} mutants (Fig. 1B). The spatial differentiation of the retinal neuroblastic layers was similar in the two embryo types, and cell counts in the INL and ONL of the mutants were, respectively, 14.9 ± 1.3 and 56.0 ± 3.9 (mean \pm SEM of cells per field). These were not significantly different ($P < 0.05$) from the 18.4 ± 1.6 and 63.9 ± 2.5 , respectively, counted in the wild-type INL and ONL (Table 1). TEM analysis also revealed that the retinal photoreceptor cells and their synapses to the RPE were similar in the wild-types and the *Btrc*^{-/-} mutants (Fig. 2). Taken together, these data indicate that deletion of the *Btrc* gene has no recognizable effect on the embryonic retina's basic spatial organization.

Cholinergic Amacrine Cells Are Absent From the *Btrc*^{-/-} Retina

Because the *Btrc* gene was found to be down-regulated in our previous microarray analysis of the retina in mouse embryos specifically lacking CACs as a result of the absence of skeletal musculature and consequently of FOMs, we also assessed the status of CACs in the *Btrc*-deletion mutant retina. As seen in Figure 2, cells of the typical "starburst-like" type characterized by cytoplasm elongated toward the vitreous body and containing numerous organelles (Kablar, 2003) were clearly evident under the TEM in the INL of the wild-type retina. However, no such cells could be identified in the *Btrc*^{-/-} mutant (Fig. 2). In addition, immunohistochemistry for acetylcholinesterase (AChE) indicated the presence of AChE-positive cells in the INL of the wild-type retina but not in the mutant retina (Fig. 3). Similarly, we were unable to identify CACs employing either AChE histochemistry or immunohistochemistry for choline acetyltransferase (ChAT) (Kablar, 2003; and data not shown). These data indicate a necessity for *Btrc* in the CAC differentiation process.

Reduced Tyrosine Hydroxylase-Expressing Amacrine Cell Count in the *Btrc*^{-/-} Retina

To further investigate the effect of *Btrc* deletion on amacrine cell differentiation, a count of the other amacrine cell subtypes was made in the INL after immunostaining with specific antibodies against calretinin (CR), calbindin (CB), parvalbumin (PV), and tyrosine hydroxylase (TH), (Fig. 4 and Table 1). The population of CR-expressing cells, which

normally make up 17% of all amacrine cells (Dyer and Cepko, 2001), was similar in the control and mutant retina (5.8 ± 0.5 versus 4.6 ± 0.4 cells per field, respectively; Fig. 4A,B; Table 1). CB-expressing cells, which make up 9% of amacrine cells (Dyer and Cepko, 2001), also did not differ significantly in number in wild-type and mutant retina (3.9 ± 0.2 versus 3.3 ± 0.6 , respectively; Fig. 4C,D; Table 1). Similarly, PV-expressing amacrine cells, which normally comprise 13% of the total amacrine cell count (Dyer and Cepko, 2001), had an equal number in the control and the *Btrc*^{-/-} retina (4.4 ± 0.4 versus 3.4 ± 0.4 , respectively; Fig. 4E,F; Table 1). The *Btrc*^{-/-} retina, however, showed a deficit in TH-expressing cells, which normally comprise only about 0.5% of the amacrine total. Whereas the wild-type retina had a count of 1.0 ± 0.09 cells per field, the mutant retina had the significantly lower ($P < 0.05$) count of 0.6 ± 0.09 , (Fig. 4G,H; Table 1).

The *Btrc*^{-/-} Retina Exhibits a Reduction in Retinal Ganglion Cell Population

Next, we attempted to establish whether the absence of *Btrc* might affect RGC differentiation. For this, the retina was stained for Islet 1/2 and, as shown in Figure 4I and 4J, the number of Islet 1/2-expressing cells was significantly lower in the mutant retina than in the wild-type (9.2 ± 0.7 versus 12.3 ± 0.7 , cells per field, respectively; Table 1), suggesting that *Btrc* might indeed be involved in RGC differentiation. At the same time, this result indicates the involvement of *Btrc* in CAC differentiation, considering that Islet-1 is also present in CACs and controls their differentiation (Elshatory et al., 2007).

Btrc Deletion Reduces Amacrine Progenitor Cell Counts

To determine what effect *Btrc* deletion might have on the progenitors of amacrine cells, a count of these cells was made after staining for the amacrine cell precursor proteins syntaxin and VC1.1 (HNK-1/N-CAM). Syntaxin- and VC1.1-positive progenitor cells were found to give rise to amacrine and horizontal interneurons and rods (Barnstable et al., 1985), making these markers less optimal. In fact, the markers used in this and the following section of the study are not exclusively expressed in the cells of interest, because they label other cells that are biased but not yet committed to the certain cell fate, hindering the investigation of this issue. As depicted in Figure 5, syntaxin and VC1.1 are detectable in both the INL and the ONL, and whereas the number of syntaxin-expressing amacrine precursor cells was similar for the INL (4.4 ± 0.6 cells per field in the mutant and 5.1 ± 0.2 in wild-type), it was found to be significantly reduced in the mutant ONL (12.4 ± 1.4 versus 20.4 ± 0.5 , respectively; Fig. 5A,B; Table 1). In addition, there was a significantly lower number of VC1.1-expressing amacrine precursor cells in the *Btrc*^{-/-} retina as compared to the wild-type in both the INL (6.8 ± 0.3 versus 10.3 ± 1.1 cells per field) and the ONL (24.1 ± 2.2 versus 32.0 ± 1.5 ; Fig. 5C,D; Table 1).

Augmented Retinal Ganglion Cell Precursor Counts in *Btrc*^{-/-} Mutants

Having also noted a reduction in the number of RGCs, we subsequently stained for the RGC progenitor cell protein nestin. Nestin is a general marker for neuronal progenitor cells (Tropepe et al., 2000) and is employed here because it is expressed by the RGC precursors (Waid and McLoon, 1998). The resulting count showed that the number of nestin-expressing cells was significantly higher for both neuroblastic layers in the mutants as compared to

controls (6.2 ± 0.8 versus 3.6 ± 0.2 cells per field respectively for INL, and 20.0 ± 2.1 versus 13.8 ± 0.2 for ONL; Fig. 5E,F; Table 1).

The *Btrc*-Deletion Mutant Depicts Normal Retinal Cell Proliferation and Apoptosis

In an attempt to elucidate the possible processes underlying the observed differences in the population of various retinal cell types in the *Btrc* mutant retina, we also analyzed retinal cell proliferation and programmed cell death (apoptosis). However, we were not able to detect any cells immunopositive for proliferating cell nuclear antigen (PCNA) in any of the sections examined from either the control or the mutant retina (Fig. 6A,B). In addition, after immunostaining for activated caspase-3 (which indicates apoptotic cells), although a few occasional cells were visible on some sections, the staining appeared very low and was not perceptibly different between the wild-type and the mutant retina (Fig. 6C,D). Importantly, the current findings are consistent with our previous cell proliferation/death data (Kablar, 2003).

In summary, our results indicate that, despite having no apparent effect on basic spatial organization, cycle kinetics, or cell survival in the retina at this stage of development, by some as yet unspecified mechanism the absence of *Btrc* affects progenitor cell competence and the eventual ratio of the resulting differentiated amacrine cells as well as the amount of retinal ganglion cells.

DISCUSSION

The aim of this work was to provide a follow-up to our previously performed microarray experiments, where we found *Btrc* to be strikingly down-regulated in retinas that lack CACs (Baguma-Nibasheka et al., 2006). As previously stated, CACs are responsible for motion vision and directional selectivity (Yoshida et al., 2001 and references therein). The neural retinas of muscleless term fetuses do not contain any CACs (Kablar, 2003, and references therein). Whether this is an example of cell differentiation being dependent on its function, or simply a coincidence, we do not know at this point, and we could not address it more directly than we already have (Kablar, 2003), unless we were provided with another mouse mutant without extraocular musculature and examined its neural retina. For instance, *Pitx2* nulls apparently specifically lack the extraocular muscles and they are viable at term (Diehl et al., 2006), therefore representing an ideal occasion for testing our hypothesis.

In the meantime, we used the opportunity provided by the retinas from muscleless fetuses to assess the differences in gene expression pattern between the control and the mutant retinas employing 15K mouse cDNA microarray slides from the Ontario Cancer Institute (Baguma-Nibasheka et al., 2006). This way, otherwise inaccessible CACs have been assessed for the differences in gene expression, and the profile of genes obtained after the systematic subtractive microarray analysis approach (SSMAA) revealed some molecular players relevant to the differentiation of CACs. Our analysis discovered two molecules whose mouse mutants lack CACs: *adapter-related protein complex 3, δ 1 subunit* (*Ap3 δ 1*) and *β -transducin repeat containing* (*Btrc*) (Baguma-Nibasheka and Kablar, unpublished data).

In fact, the *Btrc*^{-/-} retina showed a normal number of cell layers and number of cells per layer with normal cell proliferation and apoptosis. However, there was a complete absence of CACs and a decrease in tyrosine hydroxylase-expressing amacrine cells. The population of other amacrine cell subtypes examined was similar to that in the wild-type retina, whereas that of the amacrine precursor cells was decreased. There was also a reduction in the number of retinal ganglion cells, whereas their progenitors were increased.

A possible mechanism of action of *Btrc* could be via the cell cycle machinery. As mentioned earlier, numerous studies have shown that, by promoting the catabolism or processing of a broad range of proteins and transcription factors at specific stages of the cell cycle, the SCF^β-TrCP complex is involved in the control of mutually very diverse processes, and in particular in the mechanisms of cell proliferation and apoptosis (Shh and IkappaB; Jensen and Wallace, 1997; Hatakeyama et al., 1999; Hattori et al., 1999; Winston et al., 1999), as well as the embryonic patterning (Gli3; Wang and Li, 2006). It is, therefore, possible that by destroying the proteins that positively regulate cell proliferation, *Btrc* causes cell differentiation to begin. Hence, in the absence of *Btrc*, in *Btrc* null mice, some cell types, which primarily depend on *Btrc* for their terminal functional maturation, will not differentiate, and that apparently includes CACs.

Unfortunately, the *Ap3δ1* mutants are not going to be very useful for further studies of CACs function, because they become blind soon after birth (Qiao et al., 2003). However, the *Btrc* nulls are viable and not blind (Nakayama et al., 2003) and, therefore, represent a model for further motion vision and directional selectivity studies, employing various approaches (e.g., electrophysiology, behavioral studies, etc.).

Additionally, via our previous microarray analysis (Baguma-Nibasheka et al., 2006) two more genes have been identified, and their knockout mice are in the EUCOMM-KOMT-NORCOMM pipeline (*protein sorting nexin 17* or *Snx17* and *WD repeat domain 5* or *Wdr5*), providing an opportunity to study the function of *Snx17* and *Wdr5* in CAC development in a conditional manner. Finally, all the identified molecules (and especially *Ap3δ1* and *Btrc*, since their mouse mutants revealed the lack of CACs) could also serve as specific markers for CACs, opening a large variety of possibilities to study the development and function of CACs in vivo, for those belonging to the field of retinal neurobiology.

EXPERIMENTAL PROCEDURES

Animal Breeding and Sample Collection

Homozygous *Btrc*-deficient fetuses were obtained by the interbreeding of heterozygous *Btrc* +/- parents as described in Nakayama et al. (2003). All fetuses were collected by Cesarean section at embryonic day (E) 18.5 and the eyeballs immediately enucleated and processed for microscopic examination. Animal use and care were in accordance with all institutional guidelines.

Immunohistochemistry and Morphometry

Immunohistochemistry was performed as previously described (Kablar, 2003) on paraffin-embedded 4µm sections. Hematoxylineosin staining followed the recommended Sigma (St.

Louis, MO) kit protocol. Immunostaining used the following primary antibodies: Sigma mouse monoclonals against calbindin, 1:200; syntaxin, 1:500; and HNK/NCAM VC1.1, 1:2,000; Novocastra mouse monoclonals against acetyl-cholinesterase (AChE), 1:150 and tyrosine hydroxylase, 1:40; Developmental Studies Hybridoma Bank mouse monoclonals against Islet 1/2, 1:40 and nestin, 1:4; Dako mouse monoclonal against proliferating cell nuclear antigen (PCNA), 1:400 and Chemicon mouse monoclonal against parvalbumin, 1:1,000 and rabbit polyclonals against calretinin, 1:500 and activated caspase-3, 1:100. Secondary antibodies were from Zymed (amino ethyl carbazol [AEC] staining) and Molecular Probes (fluorescent staining), and hematoxylin nuclear counterstaining followed the AEC immunostains. In morphometry, the cells for each animal (3 wild-type and 3 mutant embryos) were averaged from counts in seven randomly selected 2,500 μm^2 fields on each meridional serial section (every 10th section was considered) of the complete eyeball in the manner that is extensively described in Kablar (2003). The magnification used to count and to generate the insets was 1,000 \times .

Transmission Electron Microscopy (TEM)

For electron microscopic analysis, eyeballs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), post-fixed in 1% osmium tetroxide, and dehydrated in an ascending series of ethanol before embedding in LR White acrylic resin. The LKB ultramicrotome was used to make 100-nm-thick sections, which were then placed on 300-mesh copper grids and stained with 2% aqueous uranyl acetate and lead citrate. The samples were examined in a JEOL JEM 1230 electron microscope equipped with a Hamamatsu ORCA-HR digital camera.

Statistical Analysis

The samples used for morphometric analyses were from three wild-type and three *Btrc*^{-/-} fetuses. The resulting counts were compared using the Student's *t*-test, with differences of *P* < 0.05 considered significant. Data are presented as mean \pm standard error of the mean (SEM).

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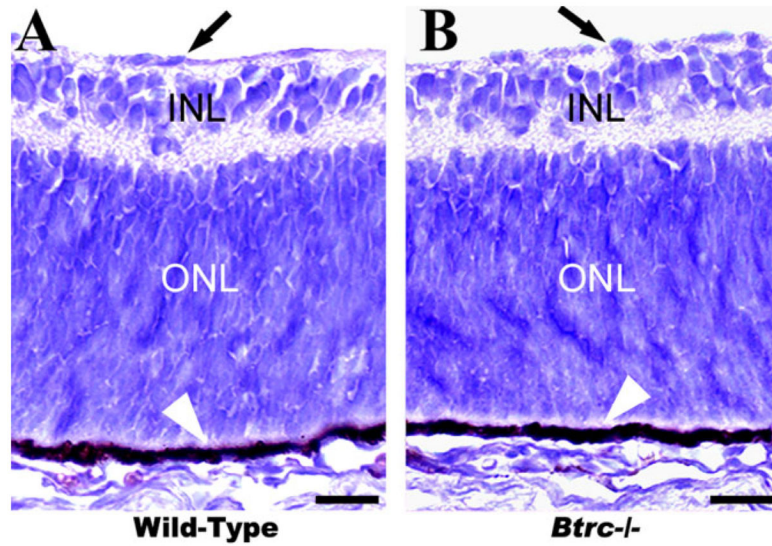


Fig. 1. The basic spatial organization of the mutant *Btrc*^{-/-} retina appears normal. On hematoxylineosin-stained paraffin-embedded sections, the inner and the outer nuclear (or neuroblastic) layers (INL and ONL, respectively) appear indistinguishable between the wild-type (A) and *Btrc*^{-/-} (B) embryonic retina. Arrows indicate the retinal inner limiting membrane. Scale bar = 25 μ m.

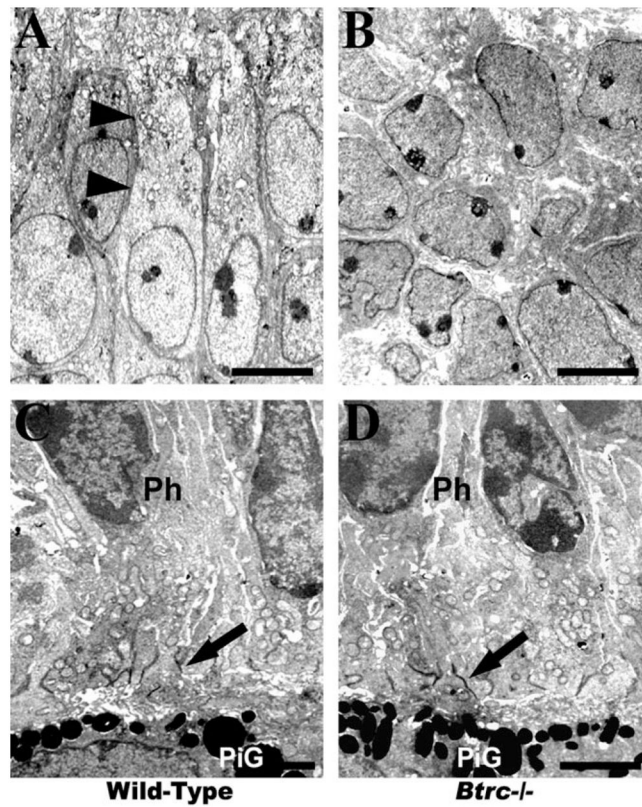


Fig. 2. Electron microscopy reveals the absence of cholinergic amacrine cells in the *Btrc*^{-/-} null retina. The “starburstlike” cell type (arrowheads) characterized by cytoplasm elongated toward the vitreous body and containing numerous organelles is identifiable in wild-type (**A**) but not the *Btrc*^{-/-} mutant retina, where most cells appear to be undifferentiated neuroblasts (**B**). The morphology of the photoreceptor cells (Ph) and their synapses with the retinal pigment epithelium (arrows in **C** and **D**), however, was not different in the two types of retina. Note the pigment granules (PiG) in the pigment epithelium. Scale bar = 4 μm (A,B) (original magnification, $\times 4,000$) and 2 μm (C,D) ($\times 8,000$).

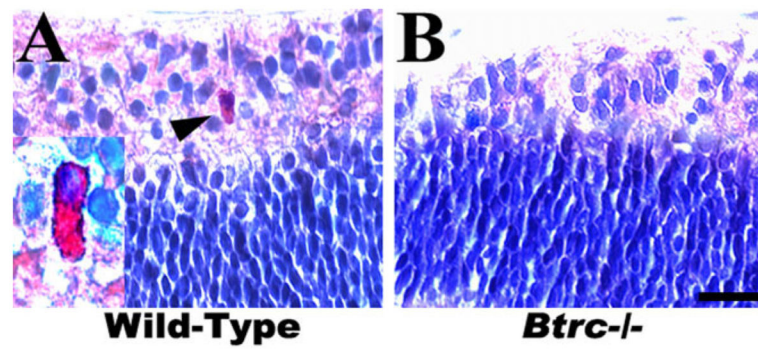


Fig. 3. The mutant retina lacks cholinergic amacrine cells. Immuno-histochemistry indicates the presence of acetylcholinesterase-positive cells (arrowhead and inset) in the inner nuclear layer of the wild-type (**A**) but not the mutant retina (**B**). Scale bar = 25 μ m.

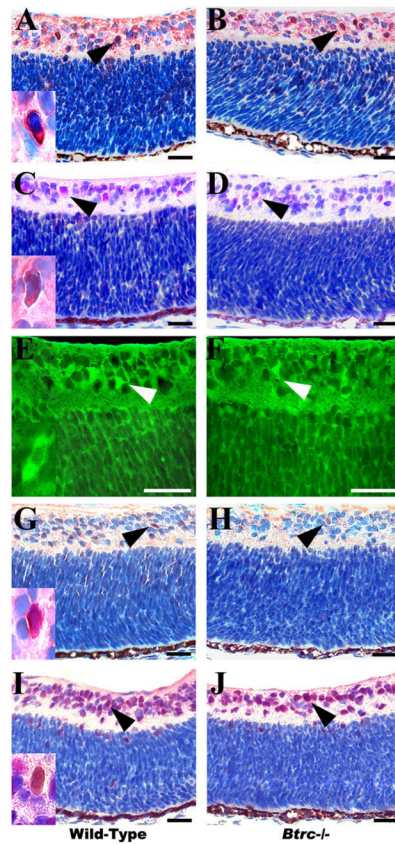


Fig. 4. Aberrant amacrine and ganglion cell numbers in the *Btrc*^{-/-} retina. Immunohistochemistry against the cytoplasmic markers calretinin (**A,B**), calbindin (**C,D**), parvalbumin (**E,F**), and tyrosine hydroxylase (**G,H**), (arrowheads) reveals a decrease in tyrosine hydroxylase-expressing amacrine cells in the *Btrc*^{-/-} retina (**H**), whereas the population of other amacrine cell subtypes appears similar to that in the wild-type retina. The number of retinal ganglion cells, indicated by the nuclear marker Islet 1/2, was also lower in the mutants (**J**) than in the wild-type (**I**). Insets are higher magnifications of positively stained cells. Scale bar = 25 μ m.

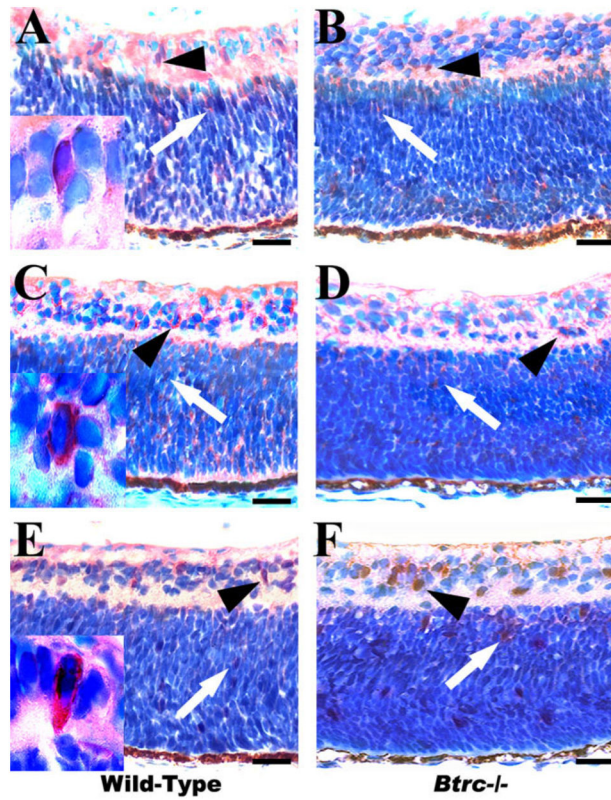


Fig. 5. Differences in precursor cell populations in the *Btrc*^{-/-} retina. The number of cells immunopositive for syntaxin was higher in the wild-type retina (**A**) than in the *Btrc*^{-/-} mutant (**B**), as was the case for VC1.1 (**C** vs. **D**, respectively). In contrast, the number of retinal ganglion progenitor cells, signaled by nestin staining, was lower in the wild-type (**E**) than in the mutants (**F**). Arrowheads and arrows, respectively, indicate immunolabeled cells in the inner and outer nuclear layers. Scale bar = 25 μ m.

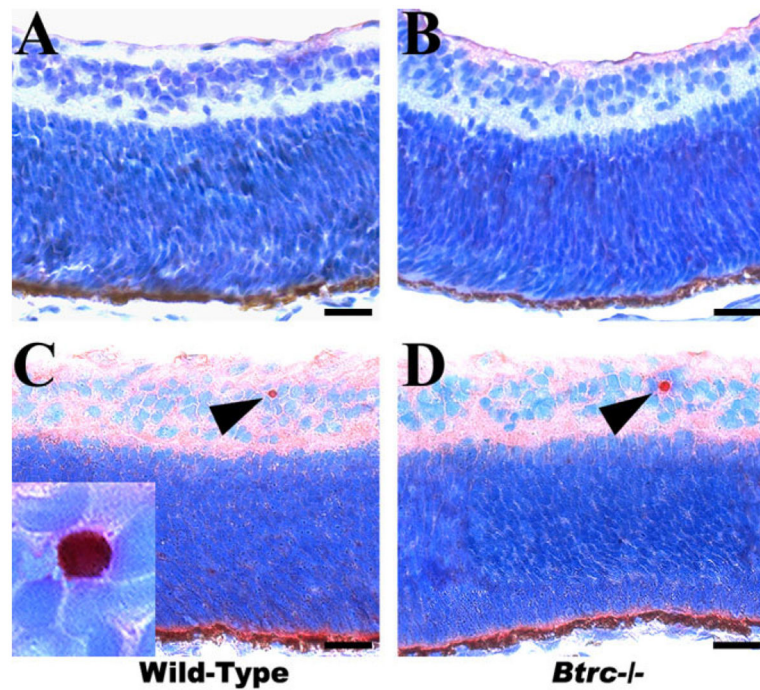


Fig. 6. Normal cell proliferation and apoptosis in the mutant retina. Cell proliferation, indicated by immunostaining for proliferating cell nuclear antigen, appears similarly negligible in the wild-type (A) and *Btrc*^{-/-} (B) retina. Similarly, staining for activated caspase-3, indicating apoptosis, was indistinguishable between the two (C and D). Arrowheads and high-power inset indicate positively stained cells. Scale bar = 25 μ m.

TABLE 1

Neuroblastic Layer Cell Counts^a of Wild-type and *Btrc*^{-/-} Retina at E18.5

		Wild-type	<i>Btrc</i> ^{-/-}
Total Cell Count	Inner	18.4 ± 1.6	14.9 ± 1.3
	Outer	63.9 ± 2.5	56.0 ± 3.9
Calretinin	Inner	5.8 ± 0.5	4.6 ± 0.4
Calbindin	"	3.9 ± 0.2	3.3 ± 0.6
Tyrosine Hydroxylase	"	1.0 ± 0.09	0.6 ± 0.09*
Parvalbumin	"	4.4 ± 0.4	3.4 ± 0.4
Islet 1/2	"	12.3 ± 0.7	9.2 ± 0.7*
Syntaxin	Inner	5.1 ± 0.2	4.4 ± 0.6
	Outer	20.4 ± 0.5	12.4 ± 1.4*
VC1.1	Inner	10.3 ± 1.1	6.8 ± 0.3*
	Outer	32.0 ± 1.5	24.1 ± 2.2*
Nestin	Inner	3.6 ± 0.2	6.2 ± 0.8*
	Outer	13.8 ± 0.2	20.0 ± 2.1*

^aMean ± SEM of cells per field.* significantly different from wild-type; *p*<0.05, n=3.